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Effects of Paramagnetic Shift Reagents on the ^{13}C Nuclear Magnetic Resonance Spectra of Egg Phosphatidylcholine Enriched with ^{13}C in the *N*-Methyl Carbons†

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ABSTRACT: Effects of paramagnetic shift reagents on the ^{13}C NMR spectra obtained from single-walled vesicle dispersions of egg phosphatidylcholine enriched with ^{13}C in the *N*-methyl carbons are investigated. Spectra obtained at 25.1 MHz show that, at Yb^{3+} to phospholipid molar ratios as low as 0.06, complete resolution of the *N*-methyl carbon resonances is obtained from molecules on the inner and outer faces of the vesicle bilayer. No precipitation of the vesicles is caused by Yb^{3+} at these concentrations nor is appreciable line broadening observed. Other paramagnetic shift reagents frequently used in proton NMR investigations of phosphatidylcholine vesicles do not give complete separation of the *N*-methyl ^{13}C signals from the two bilayer surfaces. $K_3Fe(CN)_6$, Eu^{3+}, and Pr^{3+} cause precipitation of the phosphatidylcholine vesicles at concentrations which give only incomplete resolution of these signals. T_1 measurements of the resonances separated by Yb^{3+} indicate that the choline groups on the inner bilayer surface are less mobile than are the same groups in the outer surface. Gated proton decoupling measurements, which show that the nuclear Overhauser effect is 2.8 ± 0.1, indicate that the dominant mode of relaxation is dipolar interaction.

Phospholipid vesicles are generally accepted as useful models for the study of lipid-lipid interactions of the types occurring in biological membranes. Among the physical techniques that may be utilized in such studies, nuclear magnetic resonance (NMR) spectroscopy has proved to be particularly powerful. Two NMR parameters yielding important information are spin-lattice relaxation times (T_1) and chemical shifts produced by paramagnetic shift reagents. The former can be directly related to molecular motion parameters and the latter allow analysis of the distributions of phospholipid molecules on the inner and outer surfaces of vesicles. Although many studies on the phospholipid bilayer system have been carried out using proton and ^{31}P NMR, relatively little work has been done with the ^{13}C nucleus (for a review, see Lee et al., 1974). ^{13}C NMR has several important advantages; the chemical shift range is large compared with ^{1}H and interpretation of the T_1 relaxation data is less complicated. The T_1 times of protonated carbons are dominated by the motions of the intermolecular C-H dipole (Allerhand et al., 1971). With ^{31}P NMR the situation with regard to relaxation times is more complex. Not only are the relaxation times much longer but, in phospholipid vesicles, chemical shift anisotropy makes a significant contribution to the relaxation (Berden et al., 1974). In addition, the relative isolation of the protonated ^{13}C nucleus decreases the perturbation of the chemical shift reagent on the relaxation behavior. However, these advantages of ^{13}C NMR are compromised by the low natural abundance of this nucleus compared with both ^{1}H and ^{31}P. This situation can be greatly improved, however, by synthetic enrichment of ^{13}C at specific locations in the phospholipid molecule. Enrichment markedly increases the sensitivity and in addition simplifies the spectrum to give a single resonance (Sears et al., 1974). In the study reported herein we utilize single-walled vesicles prepared from phosphatidylcholine enriched in the *N*-methyl carbons with ^{13}C (90%) to compare the effectiveness of various paramagnetic shift reagents and determine T_1 values for the *N*-methyl resonances arising from the inner and outer surfaces of the vesicle wall. In addition paramagnetic Yb^{3+} and Pr^{3+} are used to determine the ratio of resonances associated with the outer and inner surfaces of the bilayer comprising the vesicle wall (Sears et al., 1975).

Experimental Section

Preparation of ^{13}C-Enriched Phosphatidylcholine. Phosphatidylcholine enriched in the *N*-methyl carbons with ^{13}C was prepared by the condensation of phosphatidic acid derived from egg phosphatidylcholine with ^{13}C-enriched choline acetate. The [^{13}C]choline was prepared by adding 6.7 mmol of [^{13}C]methyl iodide (Merck, Sharpe and Dohme) to 8 mmol of redistilled 2-dimethylethanolamine (Aldrich) in 20 ml of dry benzene. A precipitate formed immediately.

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22901. *Received September 5, 1975.* This investigation was supported by U.S. Public Health Service Grant GM-14628. One of us (B.S.) was the recipient of a National Institutes of Health Postdoctoral Fellowship.

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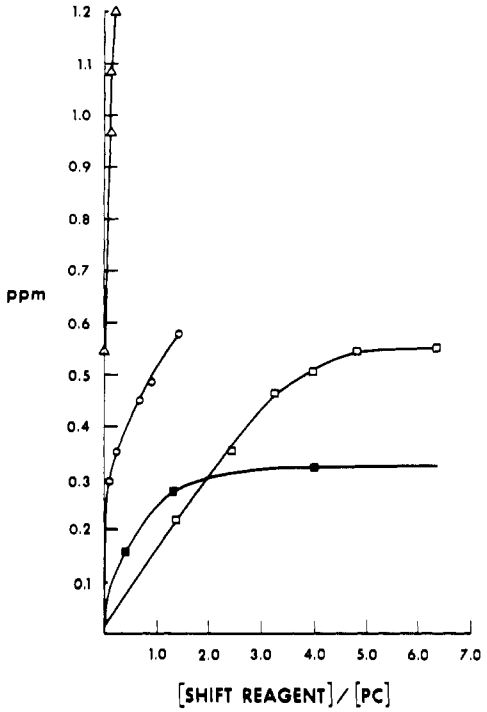

FIGURE 1: Chemical shifts produced by paramagnetic ions in the *N*-methyl ^{13}C resonance of phosphatidylcholine in the outer surface of the vesicle wall. (△) Yb^{3+}; (⊙) Pr^{3+}; (⊡) $Fe(CN)_6^{3-}$; (■) Eu^{3+}. The ordinate is the molar concentration ratio of shift reagent to phosphatidylcholine (PC). The shift is in absolute parts per million. The shifts of Yb^{3+} and Eu^{3+} are upfield; the others are downfield from Me_4Si.

The reaction was allowed to proceed for 2 days at room temperature in a sealed flask. Filtration of the [^{13}C]choline iodine gave a yield of 95% (6.5 mmol). [^{13}C]Choline iodine was converted to the acetate salt by passage through an anion exchange column (IR-45) previously equilibrated with acetate. Nearly quantitative recovery of the choline from the exchange column was achieved. Choline concentrations were estimated by the method of Sass et al. (1958).

Purified phosphatidylcholine was prepared from hen eggs following procedures previously described (Litman, 1973). Phosphatidic acid was prepared from the phosphatidylcholine using phospholipase D according to the method of Dawson (1967). One gram of phosphatidylcholine was taken to dryness and then dispersed in 200 ml of a buffer containing 0.225 M $CaCl_2$ and 0.017 M sodium acetate (pH 5.4). Then, 100 mg of cabbage phospholipase D (Sigma) was added to the mixture. Finally, 40 ml of ether was added, and the vessel was stoppered and incubated at 32 °C for 4 h. At the end of the reaction the mixture was extracted four times with diethyl ether. The ether was evaporated to dryness and the residue was extracted by the Bligh-Dryer procedure with 5 mM EDTA[1] in the aqueous phase. The phosphatidic acid gave a single spot on silica gel thin-layer chromatography using 65:25:4 ($CHCl_3$:CH_3OH:H_2O) when stained with I_2 vapor. No phosphatidylcholine was detected. The concentration of phosphatidic acid was estimated by the method of Gomori (1942).

Condensation of [^{13}C]choline acetate and phosphatidic acid was accomplished using 2,4,6-triisopropylbenzenesulfonyl chloride (Aldrich) as a coupling reagent according to the method of Aneja and Chadha (1971). The resulting [^{13}C]-*N*-methylphosphatidylcholine was isolated by silicic acid chromatography. The yield based on the starting amount of egg phosphatidic acid was 50%.

Vesicle Preparation. [^{13}C]-*N*-Methylphosphatidylcholine (160 μmol) was lyophilized from benzene. Four milliliters of pD 8.4 buffer containing 0.1 M KCl and 0.01 M Tris in D_2O (Sigma, 99.8%) was added to the dry phospholipid. This suspension was then sonicated for 30 min under nitrogen at 4 °C using a Branson W-350 sonicator. The dispersion was centrifuged at 10 000*g* for 10 min to remove titanium fragments and undispersed lipid. The final phosphatidylcholine concentration was 0.038 M as determined by phosphate analysis.

NMR Spectroscopy. All measurements were conducted at 23 °C using a JEOL PS-100P/EC-100 Fourier transform spectrometer operating at 25.1 MHz. The 90° pulse width was calibrated at 20 μs. Complete proton decoupling was accomplished using a phase modulated noise decoupler. The nuclear Overhauser effect (NOE) was measured using gated proton decoupling (Freeman et al., 1972). The decoupler was off for at least five T_1 values in the gated experiments. Typical conditions were a spectral width of 5.0 kHz using 4K data points in the frequency domain. An excellent signal-to-noise ratio was achieved after averaging 100 transients. Paramagnetic shift reagents were added to the vesicle dispersion from stock solutions (1.0 M $YbCl_3$, $EuCl_3$; 0.5 M $PrCl_3$, $K_3Fe(CN)_6$). The chemical shift produced by the paramagnetic ion was determined by measuring the separation between the *N*-methyl carbon resonances arising from the inner and outer surfaces of the vesicle wall.

T_1 values were determined using the 180°-τ-90° pulse sequence (Vold et al., 1968). The usual precautions were taken (Farrar and Becker, 1971). At least 10 τ values were used in the least-squares analysis of the data. In all cases, the linear correlation coefficient plot of ln $[(A_0 - A_\tau)/2A_0]$ vs. τ was greater than 0.995. The T_1 values have a nonsystematic error of ±5%. The areas of the separated resonances were determined either by planimetry or by measuring the integral height of each resonance. Similar results were obtained by both methods.

Results and Discussion

Chemical Shifts Produced by Paramagnetic Ions. Because of the more complex nature of the carbon nucleus, shift reagents which have been found useful for proton NMR studies of phosphatidylcholine vesicles may not be suitable for ^{13}C NMR work. Figure 1 shows the chemical shifts produced by four paramagnetic ions in the ^{13}C resonances arising from molecules in the outer surface of the vesicle wall. It is apparent that Yb^{3+} gives the largest shift over the concentration range examined. Although Pr^{3+} is less effective, adequate shifts are produced at relatively low praseodymium to phospholipid molar ratios. This is in contrast to $Fe(CN)_6^{3-}$ which produces useful shifts only at high concentration ratios. Eu^{3+} causes only very small shifts even at these high ratios.

Because of the low resonance frequency of the ^{13}C nucleus, a chemical shift of at least 0.9 ppm is required to obtain complete spectral resolution of the *N*-methyl carbon resonances arising from molecules on the inner and outer surfaces of the vesicle wall. In contrast to this condition, at 220 MHz proton chemical shifts as low as 0.17 ppm can cause complete separation of the two resonances (Andrews et al., 1973). It is apparent from Figure 1 that complete resolution of the two resonances is obtained with Yb^{3+} at Yb^{3+} to

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; NOE, nuclear Overhauser effect; PC, phosphatidylcholine.

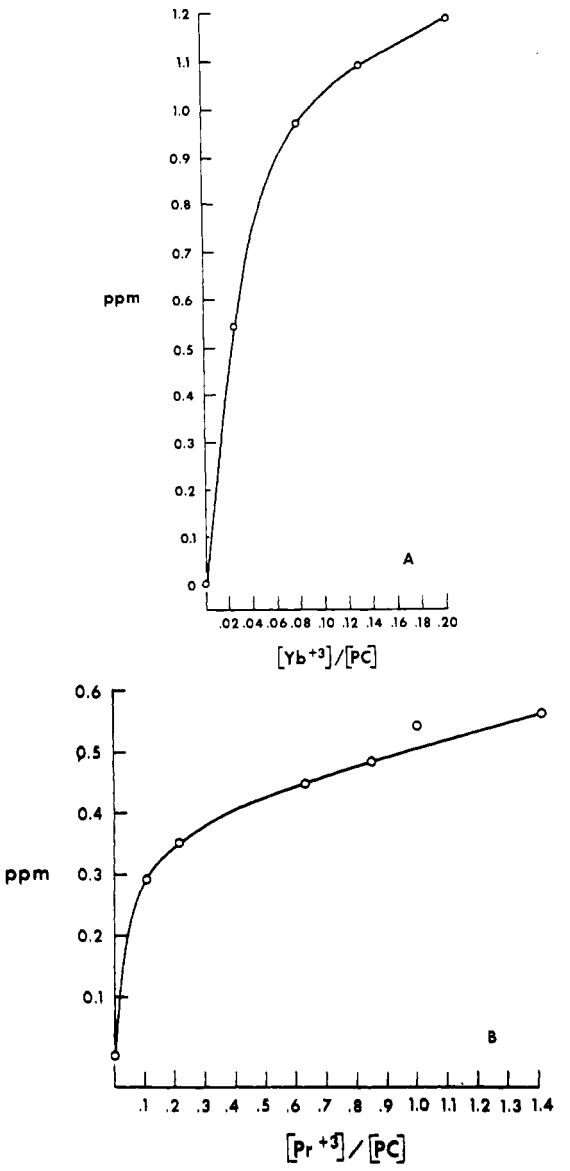

FIGURE 2: Chemical shift produced by Yb^{3+} (A) and Pr^{3+} (B) in the *N*-methyl ^{13}C resonance of phosphatidylcholine in the outer surface of the vesicle wall. The shift is in absolute parts per million. The shift of Yb^{3+} is upfield, and of Pr^{3+} is downfield from Me_4Si.

phospholipid molar ratios above 0.06 whereas the resolution is never complete with the other three ions. In addition Yb^{3+} gives signals with only slight broadening. The fact that Yb^{3+} is the most efficient paramagnetic shift reagent in the group of four ions studied arises from the large pseudocontact/contact chemical shift ratio of this lanthanide toward carbon (Gansow et al., 1973). This behavior has been noted for organic systems by others (Chadwick and Williams, 1974; Kessler and Matter, 1974).

All four shift reagents at high concentrations cause aggregation and eventual precipitation of the vesicles. With Yb^{3+}, however, complete spectral resolution can be obtained at concentration ratios which do not cause aggregation. Pr^{3+} causes some turbidity in the concentration range studied. Vesicle aggregation problems encountered with Eu^{3+} and $Fe(CN)_6^{3-}$ are severe. Further studies with these two ions were not carried out.

Figure 2 depicts on an expanded scale the data presented for Yb^{3+} and Pr^{3+} in Figure 1. It is apparent that there is a saturation effect of the metal ion on peak separation. As

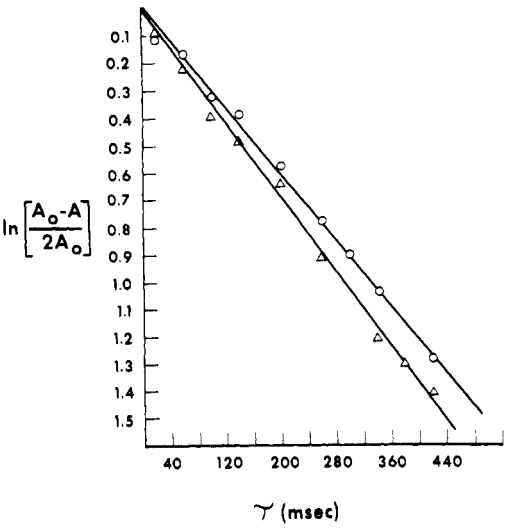

FIGURE 3: Plot of ln $[(A_0 - A_\tau)/2A_0]$ vs. τ, the waiting period between the 180° and the 90° pulse for the outer (⊙) and inner (△) surface *N*-methyl carbons.

Table I: T_1 Times of Resonances Arising from *N*-Methyl ^{13}C on Inner and Outer Vesicle Surfaces.

Sample	T_1 (ms)	[Lanthanide^{3+}]/[PC]
Phosphatidylcholine[a]	330 ± 15	
Phosphatidylcholine + Yb^{3+}		
Inner surface	300 ± 15	0.08
Outer surface	330 ± 15	0.08
Phosphatidylcholine + Pr^{3+}		
Inner surface	300 ± 15	0.63
Outer surface	300 ± 15	0.63

[a] Phosphatidylcholine concentration, 38 mM.

Huang and co-workers have noted this type of behavior suggests two classes of metal–lipid binding sites: one consisting of a few high affinity sites, the other consisting of a larger number of weaker binding sites (Huang et al., 1974). With both Yb^{3+} and Pr^{3+} only 5–10% of the total phospholipid constitutes high affinity binding sites.

Effect of Yb^{3+} and Pr^{3+} on T_1. Enrichment of a particular carbon atom with ^{13}C permits determination of T_1 times with ease and accuracy. Even at the relatively low vesicle concentration of 38 mM, a good signal-to-noise ratio is obtained in less than 2 min. T_1 values are summarized in Table I. Phosphatidylcholine vesicles with no paramagnetic ion added yield a T_1 time of 330 ± 15 ms. At a Yb^{3+}/phosphatidylcholine ratio of 0.08, the *N*-methyl carbon resonances arising from the inner and outer surfaces of the vesicle wall are clearly separable. T_1 times for the outer surface resonances in the presence of Yb^{3+} are similar to those obtained for vesicles without added paramagnetic ion. It should be noted, however, that the T_1 times arising from the inner surface where there is no Yb^{3+} are slightly smaller than the value obtained for the outer surface resonance. Since the measurements of both signals are made simultaneously on the same sample, the difference is not due to an uncertainty in T_1 values. A typical plot of ln $[(A_0 - A_\tau)/2A_0]$ vs. τ is presented in Figure 3. During the course of the experiments it was always possible to see on the recorder plots that the inner surface resonance nulled before the

outer surface resonance. T_1 values for the outer and inner surface resonance obtained with Pr^{3+} are, however, identical. This is probably due to the fact that the high concentrations of Pr^{3+} required to obtain effective separation of the two resonances cause a reduction in the T_1 time of the outer resonance.

Kornberg and McConnell (1971) have reported that the molecular rotational motions of phosphatidylcholine molecules on the inner surface of the vesicle wall are markedly more restricted than are those of molecules on the outer surface. This result was obtained with an electron spin resonance probe produced by coupling a nitroxide group to the choline nitrogen of phosphatidylcholine. Levine and coworkers utilizing proton NMR and Eu^{3+} as a shift reagent have found that the inner surface molecules are only slightly less restricted than those on the outer surface as assessed by T_1 times (Levine et al., 1973). In contrast, however, is the report of Radda and co-workers using $Fe(CN)_6^{3-}$ as a shift reagent and proton NMR that the inner surface molecules are less rotationally restricted than are molecules on the outer surface (Berden et al., 1975). One possible explanation for this disagreement may be the amount of shift reagent added to the vesicles. As Berden et al. point out, the concentration of the $Fe(CN)_6^{3-}$ used in their studies caused a 10–20% reduction in the T_1 times for the outer surface resonances. We have already observed a similar reduction in the T_1 times when Pr^{3+} as compared with Yb^{3+} is used as the shift reagent. Since the observed differences in the T_1 times between the inner and outer surface are not large to begin with, this observed reduction in the outer surface T_1 times is large enough to account for the results of Berden et al. The results shown in Table I clearly indicate that rotational mobilities of the molecules on both bilayer surfaces are very similar. The data obtained with Yb^{3+} at low ion to phospholipid ratios suggest that the inner surface molecules are more restricted than are those on the outer surface in agreement with results of Levine et al. (1973) and Kornberg and McConnell (1971).

It is possible that the different motional freedom of molecules in the two faces of the vesicle wall is due to different molecular packing densities in the two surfaces which are the consequence of the very small vesicle diameter (Sheetz and Chan, 1972; Thompson et al., 1974). Not only is the radius of the outer face (105 Å) almost twice the radius of the inner face (65 Å), but the signs of the two radii are different. Thus relative to the bilayer wall, the outer surface is defined by a positive radius, the inner surface by a negative radius. This configuration causes the volume of bilayer subtended by unit area on the outer surface to decrease in cross section toward the center of the vesicle wall, whereas the volume subtended by unit area on the inner surface increases in cross section as distance toward the center of the bilayer decreases. These geometrical considerations lead to the conclusion that the polar head groups of constituent phosphatidylcholine molecules may be expected to be more restrictively packed on the inner bilayer surface than on the outer (Sheetz and Chan, 1972; Thompson et al., 1974).

Ratio of Resonance Intensities Associated with the Outer and Inner Vesicles Surfaces. Bystrov et al. (1971) first showed with proton NMR that paramagnetic shift reagents can be used to estimate the relative proportions of phosphatidylcholine molecules occupying the outer and inner surfaces of the vesicle wall. This is done by determining the ratio of the integrated intensities of the *N*-methyl resonances which are shifted by a paramagnetic shift reagent in the external aqueous phase (outer surface resonance) to those which are not (inner surface resonance). Studies of this type have been carried out not only with vesicles comprised solely of phosphatidylcholine but also with vesicles comprised of phosphatidylcholine and cholesterol (Huang et al., 1974), or phosphatidylglycerol (Michaelson et al., 1973; Andrews et al., 1973), or phosphatidylethanolamine (Andrews et al., 1973; Michaelson et al., 1974).

Table II: *N*-Methyl ^{13}C Resonances. Ratio of Resonance Intensities Arising from Outer and Inner Vesicle Surfaces with and without NOE.

Sample	[Lanthanide^{3+}]/[PC]	Resonance Ratio
Phosphatidylcholine[a,b] + Yb^{3+}		
Full NOE	0.40	2.1 ± 0.1
Attenuated NOE	0.40	2.0 ± 0.1
Phosphatidylcholine + Pr^{3+}		
Full NOE	1.4	2.1 ± 0.1
Attenuated NOE	1.4	2.1 ± 0.1

[a] NOE was 2.8 ± 1 for phosphatidylcholine vesicles without lanthanide. [b] Phosphatidylcholine concentration 14–38 mM.

Table II presents the result of this type of determination utilizing the *N*-methyl ^{13}C resonances and Yb^{3+} or Pr^{3+} as shift reagents. The resonance intensity ratio obtained with both ions is 2.1 ± 0.1. Similar values close to 2 have been reported by others using proton NMR (Huang et al., 1974; Berden et al., 1975). Recent measurements of the outer radius of this type of vesicle give values very close to 105 Å (Huang and Lee, 1973; Goll et al., 1975). If the bilayer thickness is taken to be 40 Å (Wilkins et al., 1971), the ratio of the areas of the external to the internal faces of the vesicle wall is 2.6. This is the expected value of the resonance intensity ratio, if the packing density of the phosphatidylcholine in each face of the bilayer is the same, and if all *N*-methyl carbons contribute fully to the NMR signals. The lower experimental value of 2.1 could be the result of (a) a non-uniform packing density, (b) a non-uniform size distribution of the vesicles, or (c) the failure of all *N*-methyl carbons to contribute fully to the NMR signal. Although as discussed above, the difference in T_1 values as well as other data suggest that the molecular packing density in the two faces of the bilayer is in fact different, it is difficult to believe that this difference could be large enough to account for the markedly lower resonance intensity ratio. The ratio of the outer to inner resonances is expected to be very sensitive to the size distribution of the vesicles. Therefore a nonhomogeneous size distribution of the phospholipid vesicles would result in a lowering of the outer to inner surface ratio. However, this explanation is doubtful for two reasons. First, it has been shown by ^{31}P NMR that fractionation of sonicated vesicles on a Sepharose 4B column does not substantially alter the ratio of the outer to inner surface (Berden et al., 1975). Secondly, it has been shown that high-speed centrifugation of the sonicated vesicles results in a homogeneously sized population (Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., and Carlson, F., manuscript in preparation). In our experiments, the ratio of the outer to inner surface resonances was invariant whether

a low- or high-speed centrifugation was used to prepare the vesicles. The explanation for the low ratio must rest in the failure of all *N*-methyl carbons to contribute to the signal. This could have two causes: (1) a differential NOE; and (2) failure of the shift reagent to interact with all outer surface *N*-methyl carbons. Unlike ^{1}H NMR, where the intensity of the signal is a direct consequence of the number of protons, the intensity of the ^{13}C signal depends on not only the number of carbon nuclei but also on the NOE that results from ^{1}H decoupling. The NOE is different for atoms in different positions within the molecule. However, in determining the resonance ratio, signals from identical *N*-methyl carbons in the two faces of the wall are employed and hence would be expected to be equally altered by NOE. It is possible, however, that exposure to the paramagnetic ion of the *N*-methyl carbons in the outer face of the bilayer could alter the NOE for these molecules and hence reduce the resonance intensity ratio. In order to examine this possibility, the resonance intensity ratio was determined without NOE by using gated proton decoupling (Freeman et al., 1972). Using this technique the NOE without added paramagnetic ion is 2.8 ± 0.1 which is close to the theoretical limit of 2.99. This value indicates that the primary mode of relaxation of the *N*-methyl carbons in the vesicle wall is dipolar (Kuhlman et al., 1970). The gated proton decoupling procedure applied to the vesicles with paramagnetic ion added gives a resonance intensity ratio of outer to inner surface signals essentially identical with that obtained with NOE (see Table II). The low value of the ratio cannot, therefore, be due to differential NOE effects. Therefore, the reason that the expected ratio of 2.6 is not achieved must be because not all of the outer surface *N*-methyl carbons are being affected by the shift reagents. Recent ^{31}P NMR results indicate that, in a phosphatidylcholine vesicle, the polar head group has a preferred orientation parallel to the surface (Yeagle et al., 1975). In such an orientation, the *N*-methyl carbon and the adjacent phosphate oxygen atoms are relatively close. The binding of a trivalent paramagnetic shift reagent will perturb this preferred orientation by binding to the phosphate oxygen atoms. If it is assumed that the surface ordering of the polar head groups is relatively strong, then only a few perturbations, such as the binding of the shift reagent, could be tolerated by the vesicle. This hypothesis might account for the observation that only 5–10% of the total phospholipid consists of high affinity sites. Since 70% of the total phospholipid is on the outer surface, then only 7–14% of the outer surface phospholipids can bind the trivalent shift reagents at any one time without causing serious disruption in the surface properties of the vesicles. Since only a relatively small percentage of the surface area is affected at any one time by the shift reagent, it is not unexpected that some of the outer surface resonances would not be affected by the binding of the shift reagent. Since those resonances would remain unshifted, the apparent ratio of shifted to unshifted resonances would decrease from the theoretical ratio of 2.6. The experimental ratio of 2.1 is probably a reflection of the surface restrictions imposed by the vesicle geometry on the binding of these perturbing ions.

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